Retroviral-mediated expression of telomerase in normal human cells provides a selective growth advantage

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Abstract. Retroviral infection of hTERT, the catalytic component of telomerase, into BJ fibroblasts (population doubling 28) resulted in reconstitution of telomerase activity, telomere maintenance, and extension of *in vitro* lifespan. The hTERT-infected cells also exhibited increased growth rate and colony forming efficiency relative to controls, while remaining contact-inhibited and maintaining a p53-mediated damage response following γ-irradiation. All single cell-derived BJ-hTERT clones grew faster than the hTERT mass cultures and maintained telomeres; however, neither telomerase activity levels nor mean telomere length correlated with the growth rate. Introduction of hTERT rescued aged BJ fibroblasts from senescence via a telomere-dependent mechanism and provided renewed proliferative potential. Collectively, our data indicate that both early and late in the cellular lifespan of human cells, ectopic expression of telomerase using a retroviral system provides a growth advantage while maintaining normal cellular characteristics.

Introduction

In vitro, normal human cells divide for 50-90 population doublings (PDs), after which time replicative senescence or mortality stage 1 (M1) is reached and cells enter an irreversible, non-dividing state (1,2). Each time a cell divides the distal ends of linear chromosomes (telomeres) shorten, a process that eventually contributes to M1 (3-8). Telomerase, a cellular ribonucleoprotein with reverse transcriptase activity, is absent in most somatic cells but essential for maintaining the integrity and length of telomeres in immortal, germ, and stem cells (9). The minimal core components of the human telomerase enzyme consists of a template RNA component hTR (human telomerase RNA) and the catalytic subunit hTERT (human telomerase reverse transcriptase) (10-12).

Since most human cells express hTR, introduction of hTERT is sufficient to reconstitute telomerase activity. Cells ectopically expressing telomerase maintain telomeres and exhibit an extension of *in vitro* lifespan without cancer-associated changed (7,8,13,14). Using a retroviral system, we now demonstrate that ectopic telomerase expression results in increased growth rate and cloning efficiency and rescues near-senescent cells from mortality stage 1. Data from our clonal analysis and studies involving expression of a dysfunctional hTERT mutant indicate that this growth advantage is dependent on telomere maintenance but does not correlate with telomerase activity levels or mean telomere length.

Materials and methods

Cell culture, retroviral infection and PD determination. BJ foreskin fibroblasts were cultured in DMEM (Gibco-BRL) with Medium 199 (Gibco-BRL) at a 4:1 ratio with 10% Cosmic Calf Serum (HyClone Laboratories, Inc.) and gentamicin (Gibco-BRL), and incubated in 5% CO_2. Cells were retrovirally infected with either pBABE-hTERT, pBABE-hTERT-HA_3 (triple hemaglutinin tagged at the C-terminus of hTERT, which renders the protein unable to act at the telomere despite having telomerase activity) (15), or vector only (pBABEpuro). As controls for evaluating p53-mediated DNA damage response and for rescuing aged cells from senescence, BJ fibroblasts were infected with human papilloma virus (HPV) E6 and E7 (pLXSN-E6/E7) or the empty vector, pLXSN. BJ fibroblast cells at PD 28 were grown to approximately 70% confluency and infected with viral supernatants as described previously (14). Selection of hTERT-, hTERT/HA_3-, or pBABE-infected BJ cells was accomplished using 800 ng/ml of puromycin (Sigma) for 4 to 5 days, while cells infected with pLXSN-E6/E7 or the pLXSN were selected with 400 μg/ml of G418 for 10-14 days.

Cells were subsequently passaged and counted in triplicate when a 50-70% confluency state was reached. The experiment was carried out for 50-123 days (depending on the cell type) and then repeated twice. The following formula was used to calculate the new PD at each passage: $\log_{10}$ [(number of cells

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counted/number of cells plated)/0.3] + last PD. For data analysis, the least squares linear regression model (Microsoft Excel) was applied to the individual population doubling rates. As a confirmatory growth measure, the MTT microtiter assay (Promega) was performed according to the manufacturer's protocol.

Clonal efficiency assay and isolation of BJ-hTERT clones. Mass cultures of BJ-hTERT (PD 37) and BJ-BABE (PD 39) were plated in triplicate (1000 cells/plate) and incubated for 18 and 23 days, respectively. The plates were then fixed with 10% formalin, stained with crystal violet, and discrete colonies were autocounted on the basis of pixel intensity using the ChemiImager 4400 Software (Alpha Innotech). The number of colonies determined by the software within the range of 1-267 pixels was used to calculate the clonal efficiency as a percentage of 1000 cells plated. Realizing that our standard incubation conditions were suboptimal for cloning control cells, additional cultures of BJ, BJ-BABE, and BJ-hTERT/HA_3 were maintained at 4% O_2 (courtesy of Dr John Wise) to obtain an adequate number of clones for the clonal efficiency experiment.

Mass cultures of BJ-hTERT, at PD 30, were seeded at clonal density and allowed to incubate at 20% O_2 for 2 weeks (with weekly medium changes). Seven single cell-derived clones at PD 54 were expanded in order to determine whether growth rate correlates with either levels of telomerase activity and/or mean telomere length (see below).

Clonal analysis of mixed cultures of BJ-hTERT and BJ cells. Uninfected BJ cells (PD 29) and BJ-hTERT cells (PD 71) were plated together at ratios of 1:10, (BJ-hTERT:BJ) at a low seeding density (i.e., 25 and 2500, respectively) to allow for clonal growth. Cultures were incubated for approximately 2 weeks. Eighteen visible clones were isolated based on size and scraped from the plate, as previously described (17) (greater than approximately 7 mm were called L, large; and less than approximately 3 mm were called S, small). The cell pellets were lysed in microfuge tubes containing a NP40 TRAP lysis buffer containing 0.1% 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (18). Protein was quantitated using a Lowry-based assay (Bio-Rad) according to the manufacturer's protocol, and then 2 µl of each sample was assayed for telomerase activity using the telomeric repeat amplification protocol (TRAP) (see below).

Telomeric repeat amplification protocol (TRAP). Telomerase activity was measured using the TRAPeze kit (Intergen), as previously described (19,20). The semi-quantitative telomerase activity, called Q, was calculated using the ratio of the intensity of the telomerase ladder to the intensity of the 36-bp internal standard. H1299, a human lung adenocarcinoma cell line, served as a positive control.

Telomere length analysis. The median telomere length was determined using terminal restriction fragment (TRF) analysis as described previously (7,17,21).

Western blot analysis of p53 following γ-irradiation. Parental BJ, BJ-hTERT and BJ-E6/E7 were seeded in duplicate on 100-mm plates at $2x10^6$ cells per dish. Twenty-four hours after plating, one of the two plates was exposed to 10 Gy γ-irradiation. Four hours after treatment, cells were lysed with standard RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) and 25 µg of total protein from each sample was separated by SDS-PAGE. Western blotting was then performed by simultaneously probing for p53 (AB-6, Oncogene Research Products) and actin (Sigma) as a loading control.

Rescue experiment and ß-galactosidase staining. BJ fibroblast cells were infected at PD 80 with pBABE-hTERT, pBABEpuro, pBABE-hTERT/HA_3, pLXSN, or pLXSN-E6/E7 (14) as described above. Since these aged cells had such a limited proliferative lifespan, no drug selection was used to obtain the infected populations. PD times were determined and applied to a least squares linear regression model as described above. To assay for cellular senescence, ß-galactosidase histochemical staining was performed as described previously (22). The percentage of positively stained cells was determined by counting three random fields of 50 cells each. Representative fields were photographed under 20x magnification.

Results

Characterization of retrovirally produced BJ-hTERT cells. BJ fibroblasts (PD 28) were infected with either the vector only (pBABEpuro) or with the catalytic subunit of telomerase (pBABE-hTERT). The stably infected BJ cell lines were termed BJ-BABE and BJ-hTERT, respectively. As shown in Fig. 1A, telomerase activity was expressed in BJ-hTERT immediately after infection and well beyond PD 100. Whereas, the negative controls, parental BJ and BJ-BABE, had undetectable telomerase activity. Of note, retrovirally infected BJ cells expressed telomerase activity at 3 to 5-fold higher levels than hTERT-transfected cells (data not shown).

Ectopic telomerase, introduced via retroviral transfer, maintains the telomeres (Fig. 1B) and extends *in vitro* lifespan (data not shown), as reported previously using transfection-based methods (7,13,14). Consistent with an earlier study (21), BJ fibroblasts stably infected with hTERT/HA_3 readily undergo senescence (data not shown). Therefore telomere maintenance, not just telomerase activity, is necessary for preventing cellular senescence.

p53-mediated DNA damage response in γ-irradiated BJ-hTERT cells. Parental BJ fibroblasts and BJ-hTERT stably transfected clones can respond to UV-B irradiation by activating p53, while BJ-hTERT clones expressing HPV16 E6 protein are unable to respond similarly because of the abolished p53-mediated checkpoints due to degradation of p53 (14,23). Here, we have analyzed our retrovirally-infected BJ-hTERT cells in terms of p53 protein levels following γ-irradiation, which, like UV, causes DNA damage. Both BJ-hTERT and parental cells showed an increase in p53 following γ-irradiation, and as predicted, BJ-E6/E7 showed complete absence of p53 protein with or without treatment (Fig. 1C). Simultaneous probing of the membrane with an actin antibody demonstrated that all samples were equally loaded (data not shown). Thus, our retrovirally produced hTERT cells are functionally similar

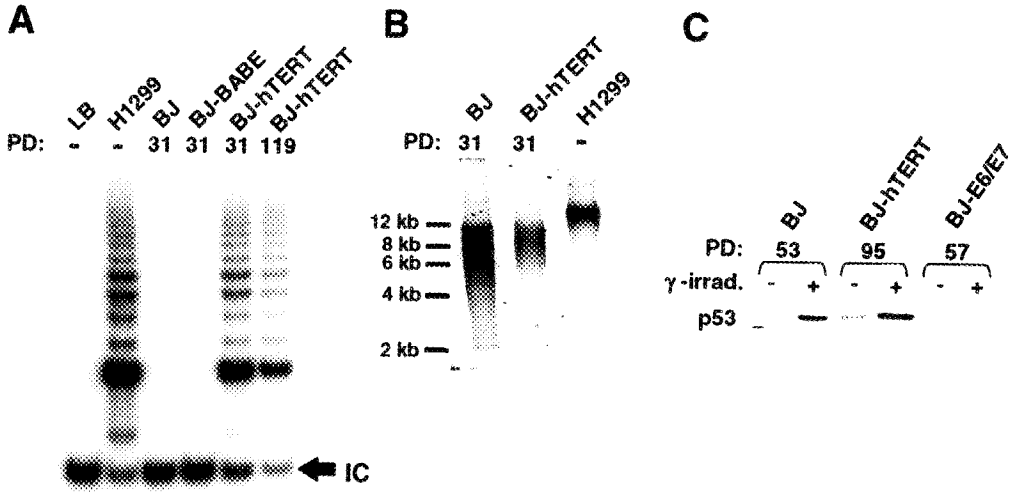

Figure 1. Retroviral introduction of telomerase maintains telomere lengths and p53-mediated DNA damage response following γ-irradiation. (A), A representative TRAP assay demonstrating ectopic telomerase activity in BJ-hTERT. A 36-bp internal standard (IC) verifies successful amplification and serves as a standard for relative quantitation. H1299, a human lung adenocarcinoma cell line, serves as a positive control, while lysis buffer (LB) is a negative control. (B), A terminal restriction fragment (TRF) assay showing telomere maintenance in the BJ-hTERT cells. (C), Western blot analysis demonstrating accumulation of p53 in parental and BJ-hTERT cells but not cells infected with HPV E6/E7 following γ-irradiation.

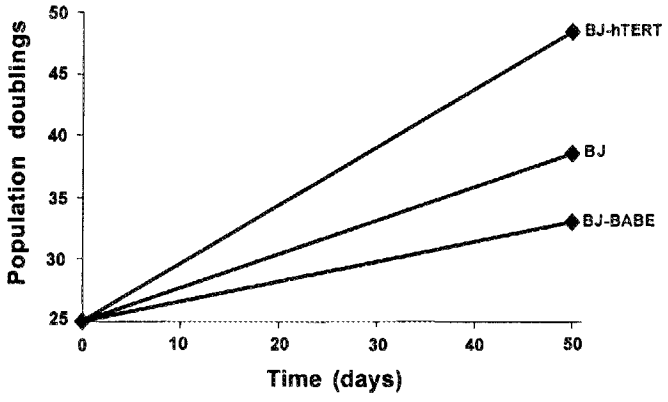

Figure 2. Expression of exogenous telomerase results in an increased growth rate. This graph represents the average growth rate over a period of approximately 50 days in the mass culture of BJ-hTERT, compared to controls, BJ-BABE and uninfected BJ cells. The slope of the line, determined by a linear regression model (Materials and methods) is a visual representation of the growth rate.

to those fibroblast lines produced using transfection-based methods. Moreover, cells expressing ectopic telomerase, whether stably introduced by infection or transfection, are distinct from cells which are immortalized following introduction of oncogenes because cell cycle check points remain intact.

Comparison of growth rates between mass populations of BJ fibroblasts with and without telomerase. In order to test whether ectopic telomerase may provide a growth advantage, we compared the average growth rate per day of uninfected BJ, and pBABE- and pBABE-hTERT-infected cells. As shown in Fig. 2, BJ-hTERT had increased population doubling rates at least 2-fold over either of the negative control populations. BJ-BABE had slightly lower growth rates in our standard incubation conditions than the uninfected control, which likely reflects detrimental cellular effects following puromycin selection. Our finding that BJ-hTERT cells were able to double more rapidly even after puromycin selection supports the notion that exogenous telomerase expression provides a growth advantage in normal cells.

Cloning efficiency in BJ-hTERT cells and controls. To further compare growth properties of BJ-hTERT cells to controls, colony-forming efficiency assays were performed. Cells at PD 39 were seeded at clonal density to assure that sufficient replicative potential remained to establish single cell-derived clones. Although all cultures exhibited a similar, high plating efficiency (data not shown), BJ-hTERT had a 21.3% cloning efficiency, while BJ-BABE had only 0.9% when incubated at 20% O_2. We observed no clones for uninfected BJ cells or for BJ-hTERT/HA_3 (data not shown). Recognizing that low oxygen is a more favorable condition for obtaining clones, BJ-BABE and uninfected BJ cells were re-seeded as above and incubated in a 4% O_2 incubator. Although discrete colonies were visible, their cloning efficiencies were still not at as high as BJ-hTERT under sub-optimal conditions. Interestingly, BJ-hTERT/HA_3 at 4% O_2 was incapable of producing any clones, indicating that expression of this tagged hTERT may pose a toxic effect in BJ cells independent of puromycin selection. The ability of hTERT-infected cells to readily form clones under unfavorable culture conditions (i.e. 20% oxygen and low seeding density) coupled with the inability of BJ controls to efficiently form clones in more optimal conditions, further supports the existence of a telomerase-mediated growth advantage.

Telomerase activity in mixed cultures of BJ-hTERT vs. BJ clones. A mixing experiment was conducted in which 10-fold fewer hTERT-infected cells were co-cultured with uninfected cells. Specifically, 2500 uninfected BJ cells at PD 29 and 250

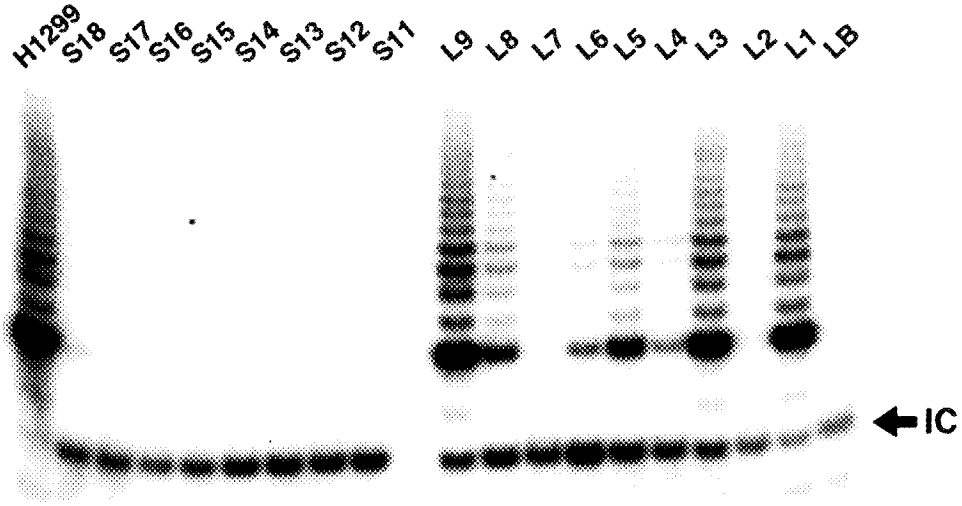

Figure 3. Comparison of telomerase activity in BJ-hTERT vs BJ clones. BJ-hTERT cells (PD 71) plated with BJ cells (PD 29) (at 1:10 BJ-hTERT to BJ) were plated together at a low seeding density and 8 small (<3 mm) and 9 large (>7 mm) clones were assayed for telomerase activity. Clones S11-18 and clones L2 and 7 have no appreciable telomerase activity; whereas clones L1, L3, L4, L5, L6, L8, and L9 all display activity, albeit at varying levels.

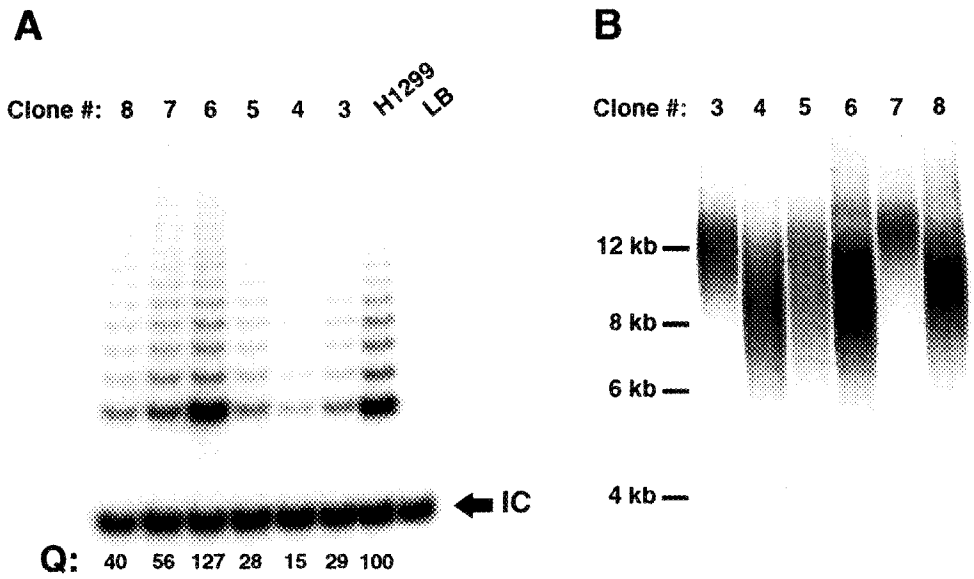

Figure 4. Comparison between telomerase activity and telomere length in BJ-hTERT clones. Clones (indicated as cl.#) at PD 54 isolated from the BJ-hTERT mass culture were tested for telomerase activity (A) and telomere length (B). Quantitative estimates are based on at least 2 independent experiments. All clones had appreciably longer telomeres than uninfected, age-matched BJ control cells (data not shown).

BJ-hTERT cells at PD 71 were mixed before plating. We reasoned that if BJ-hTERT cells have a growth advantage over uninfected BJ, the number of clones expressing telomerase should be proportionately greater than those with undetectable telomerase. Two weeks after seeding, 18 discrete clones (9 large and 9 small) were directly analyzed by TRAP. Consistent with our hypothesis, 78% of the large clones (>7 mm) were telomerase-positive while none of the small clones (<3 mm) were telomerase-positive (Fig. 3). This translates into more than 40% of the clones isolated being positive for activity with the telomerase-positive BJ-hTERT cells contributing only 9% of the cells initially seeded, suggesting a selective growth advantage for the BJ-hTERT cells even in a background of telomerase-negative cells.

Clonal analysis of growth, telomerase activity and telomere length. Having demonstrated an association between ectopic telomerase expression and a growth advantage in BJ fibroblasts, we next tested whether levels of telomerase activity and/or mean telomere length directly correlate with growth rate. For this purpose, single cell-derived BJ-hTERT clones (n=7) were expanded. All of the clones tested had telomerase activity (Fig. 4A) and maintained telomeres over the course of the experiment (>60 PDs) (Fig. 4B shows data for 6 of 7 clones; compare to the median telomere length of 6-7 kb in uninfected BJ shown in Fig. 1B). Moreover, all of the clones had noticeably higher growth rates when compared to the BJ-hTERT or BJ-BABE mass populations (Fig. 5). Data generated from the MTT colorimetric assay for determining

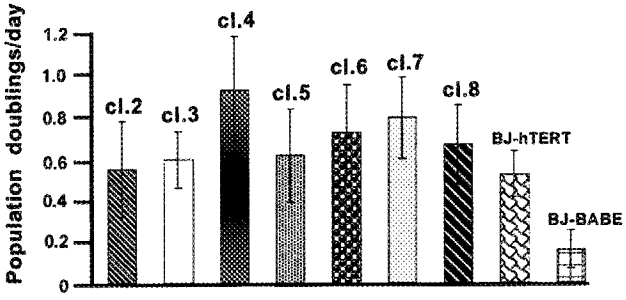

Figure 5. Elevated growth rates for BJ-hTERT clones relative to controls. Population doublings per day were calculated as detailed in Materials and methods. Values represent mean ± SD based on triplicate cultures at each data point over a period of 10 weeks.

cell growth rates for the clones and controls were highly consistent with the manual cell counts presented (data not shown). However, we failed to demonstrate a consistent, positive correlation between the levels of telomerase activity or mean telomere length and growth (Figs. 4 and 5). None of the BJ-BABE clones selected were able to proliferate beyond the initial subculturing.

Expression of telomerase rescues aged BJ cells from senescence via a telomere-dependent mechanism. We then determined whether ectopic telomerase expression is also able to provide a growth advantage to near-senescent BJ fibroblasts. Cells at PD 80 were infected with vector only (pLXSN or pBABEpuro), hTERT, hTERT/HA_3, or pLXSN-E6/E7. As shown in Fig. 6A, telomerase activity was reconstituted in near-senescent BJ cells after retroviral infection of hTERT or hTERT/HA_3 but not in those infected with pLXSN-E6/E7 or vector controls.

After a lag in growth, the hTERT and E6/E7 expressing cells showed a significant proliferative advantage over vector controls and hTERT/HA_3 cells (Fig. 6B). The E6/E7 oncogenes allow the near-senescent cells to bypass senescence by blocking the p53 and pRB pathways (24,25). However, they eventually underwent crisis at about PD 93, followed by activation of endogenous telomerase and immortalization (data not shown). BJ-hTERT has been carried out beyond PD 180 with no decrease in growth rate (data not shown). Since BJ-hTERT/HA_3 reconstitutes telomerase activity (Fig. 6A), but neither functions at the telomere (data not shown) nor rescues near-senescent cells (Fig. 6), we conclude that this selective growth advantage requires telomere maintenance.

To confirm the effectiveness of the senescence rescue, ß-galactosidase histochemical staining was performed (7,22). As shown in Fig. 7, the vast majority of BJ-BABE- and BJ-hTERT/HA_3-infected cells stained positively for ß-galactosidase and exhibited morphological features indicative of senescence, most notably an increase in cell size and volume. In contrast, BJ-hTERT and BJ-E6/E7 showed little or no positive staining and a morphology more typical of young, actively dividing fibroblasts. The following quantitative data were generated based on determining ß-galactosidase positivity in 3 random fields of 50 cells: BJ-BABE (PD 83.5), 81.3±8%; BJ-hTERT/HA_3 (PD 83.5), 78±3.4%; BJ-hTERT (PD 85.5), 4±2%; BJ-hTERT (PD 96), 2±2%; BJ-E6/E7 (PD 90), 0±0%; BJ-E6/E7 (PD 93), 11.3±4.2%. These findings indicate that late passage BJ cells can be rescued from senescence by maintaining telomeres via expression of exogenous telomerase, thus providing increased proliferative potential and a growth advantage.

Discussion

We demonstrated that expression of hTERT via retroviral infection results in reconstitution of telomerase activity, telomere maintenance, and extension of *in vitro* lifespan, as described previously for transfection-based studies (7). Novel to this study, however, was our consistent finding that ectopic

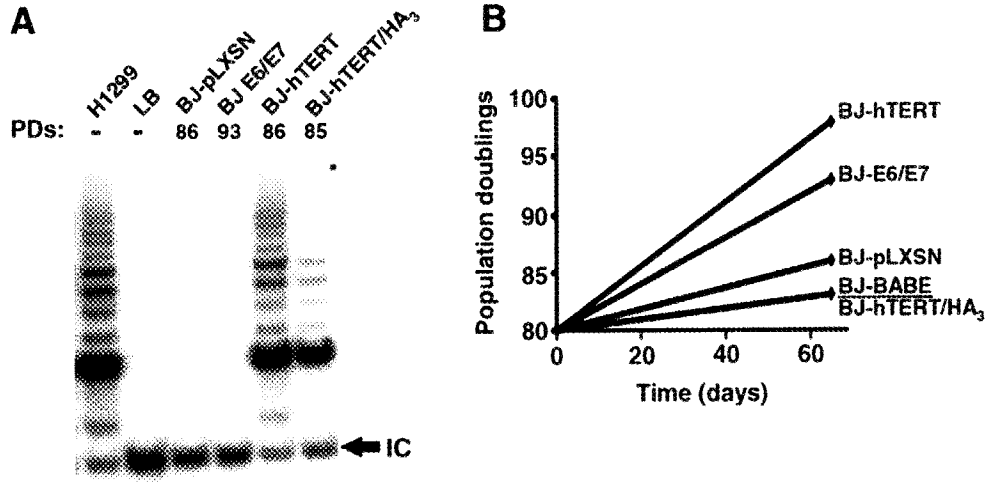

Figure 6. Expression of telomerase, via a telomere-dependent mechanism, rescues aged BJ fibroblasts from senescence. (A), A TRAP assay demonstrating reconstitution of telomerase activity in BJ-hTERT and BJ-hTERT/HA_3 cells, while activity is undetectable in parental and BJ-E6/E7 cells. (B), Linear regression plot of the relative population doublings per day over 60+ days. BJ-BABE had the same population doubling rate as BJ-hTERT/HA_3 (represented as a single line).

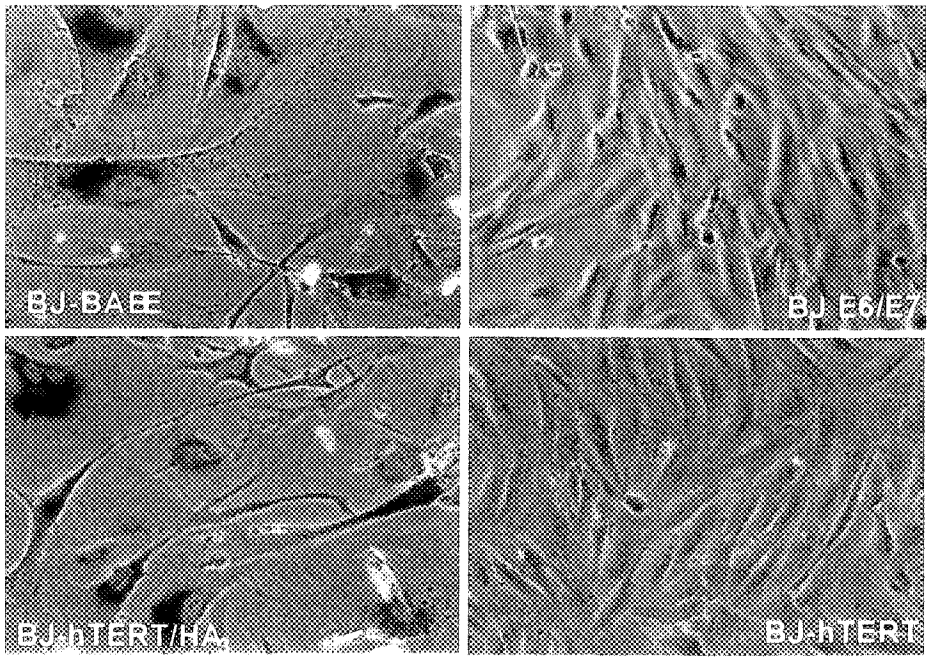

Figure 7. Absence of ß-galactosidase histochemical staining in hTERT- and E6/E7-infected BJ fibroblasts. Representative microscopic fields showing intense ß-galactosidase staining as well as classic morphological features indicative of replicative senescence only in the pBABE and hTERT/HA_3 controls. Original magnification, 20x.

telomerase expression confers a growth advantage to normal human cells. In our hands, telomerase levels tend to be higher in BJ fibroblasts following retroviral infection compared to transfection (data not shown). Therefore, we directly tested whether telomerase activity levels positively correlate with growth rate. Our extensive clonal analysis involving a comparison of growth rate, telomerase activity, and telomere lengths revealed the following: a) the relative level of telomerase activity was not predictive of mean telomere length, as similarly reported in tumor-derived clonal populations (17); b) neither telomerase activity nor mean telomere length correlated with growth rate; and c) all clones grew at a faster rate than the hTERT mass population.

To complement our studies examining the effects of ectopic telomerase expression in relatively young human cells, we have examined whether exogenous telomerase could also provide a growth advantage in the later stages of a cell's life cycle. At PD 80, a subset of BJ cells are in S-phase (a requirement for retroviral integration), however, they possess only a very limited proliferative potential. We show that following introduction of active hTERT, cells continue to divide and only rarely exhibit features indicative of senescence. Consistent with our findings, Funk *et al* (26) have recently reported that ectopic telomerase expression in aged dermal fibroblasts extends *in vitro* lifespan and prevents loss of dermal integrity associated with aging. Collectively, these data suggest that ectopic telomerase is able to provide a growth advantage to both young and old, pre-senescent human cells.

To address the mechanism by which telomerase provides this growth advantage, we have tested the ability of BJ fibroblasts infected with a hemaglutinin-tagged hTERT to form colonies following seeding at clonal density and to rescue aged cells from senescence. In both assays, we show that telomerase activity alone is not sufficient to provide a growth advantage and that telomere maintenance is required. Since cells at the end of their lifespan are challenged by the 'end replication' problem, the ability of ectopic telomerase to rescue these aged cells from senescence via a telomere-based mechanism is understandable. However, less certain is how telomerase confers a more than 2-fold growth advantage by maintaining telomeres in a relatively young cell population that has a median telomere length of 6-8 kb and is growing well. Insight may be provided by a recent study suggesting that individual, dysfunctional telomeres (i.e., those typically underrepresented, critically short telomeres within a chromosomal complement) rather than average or mean telomere length may be recognized as DNA damage and trigger a cellular response (27). Therefore, the ability of ectopic telomerase to maintain or elongate those critically short telomeres within a cell may be sufficient to provide a growth advantage. Recognizing that telomerase can heal broken chromosomes in lower eukaryotes (28,29), it is possible that ectopic telomerase may also confer a growth advantage by facilitating DNA repair. Underlying the growth advantage under suboptimal conditions (i.e., clonogenic assays at 20% O_2) may be the ability of telomerase and telomere maintenance to cause apoptotic resistance (30,31).

Besides providing a more efficient means of introducing genes than transfection, retroviral infection of telomerase also carries an additional advantage, namely, providing a growth advantage to human cells without causing cancer-associated changes. Elucidating the mechanism(s) of this growth effect may provide clues into additional cellular roles for telomerase, while retroviral delivery of hTERT into cells may also have implications for therapies involving age-related diseases and tissue engineering.

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